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The causes of evolvability and their evolution

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Abstract | Evolvability is the ability of a biological system to produce phenotypic variation that is both heritable and adaptive. It has long been the subject of anecdotal observations and theoretical work. In recent years, however, the molecular causes of evolvability have been an increasing focus of experimental work. Here we review recent experimental progress in areas as different as the evolution of drug resistance in cancer cells and the rewiring of transcriptional regulation circuits in vertebrates. This research reveals three major themes: the importance of multiple, genetic and non-genetic mechanisms to generate phenotypic diversity, of robustness in genetic systems, and of adaptive landscape topography. We also discuss the mounting evidence that evolvability can evolve, and the question of whether it evolves adaptively.

[H1] Introduction

Evolvability research is now entering its fourth decade. Although the term was first used as early as 1932, evolvability as a scientific subdiscipline of evolutionary biology is often associated with a 1989 article by Richard Dawkins[1] describing what are now called digital organisms[2]. Today, research on evolvability is integral to multiple fields, including population genetics, quantitative genetics, molecular biology, and developmental biology. Not surprisingly then, this diversity of research has led to various definitions of evolvability[3]. We here focus on one of them, because we consider it the most fundamental: Evolvability is the ability of a biological system to produce phenotypic variation that is both heritable and adaptive. The definition is fundamental because, first, heritable phenotypic variation is the essential raw material of evolution. Second, unless a biological system has the potential to produce variation that is adaptive

(beneficial) in some environments, adaptation by natural selection is impossible. Third, the definition is broad enough to apply to fields as different as population genetics and molecular biology, which study evolvability in different ways[3].

Most early evolvability research was theoretical or guided by few experimental studies[1,3-11]. This has changed. Research on evolvability is becoming increasingly experimental and driven by advances in high-throughput technologies (Box 1). The observations from such experiments are providing a mechanistic understanding of how living systems generate heritable adaptive variation[12]. We focus this Review on such experimental studies, which come from a diversity of fields, ranging from developmental to cancer biology. Many make no explicit mention of evolvability, yet they all shed light on the causes of evolvability, and some also on its evolution. They are relevant for phenomena as different as the evolution of antibiotic resistance in bacteria, and the evolutionary rescue of populations threatened by climate and other environmental change. Their insights fall into three major categories, which provide a scaffold for this Review.

The first major category encompasses molecular mechanisms that create phenotypic heterogeneity, and do so not just through DNA mutations, but even in the absence of such mutations. These mechanisms have become central to evolvability research, because they allow **isogenic populations [G]** to create phenotypic variation, some of which may facilitate survival in new or rapidly changing environments, and may thus provide time for an advantageous phenotype to be reinforced or stabilized via DNA mutation, gene duplication, recombination, or epigenetic modification. The second category of evidence revolves around robustness, which is central to evolvability, because it allows an evolving population to explore new genotypes without detrimentally affecting essential phenotypes. The resulting genotypic diversity may serve as a springboard for subsequent mutations to generate novel phenotypes, or it may bring forth new phenotypic variation when the environment changes. The third category of evidence regards the topographical features of an adaptive landscape, such as its smoothness, and a population's location within such a landscape. These factors determine the amount of adaptive phenotypic variation that mutation can bring forth. Adaptive landscapes

provide a useful geometric framework to encapsulate genotype-phenotype (or fitness) relationships that affect evolvability.

Unfortunately, space constraints prevent us from reviewing other important aspects of evolvability research, including the roles of **phenotypic plasticity [G]** , organismal development, **modularity [G]** , and **pleiotropy [G]** , as well as theoretical advances. Additionally, we frame our Review primarily around mechanisms of **pre-mutation evolvability [G]** and mechanisms that do not require genetic change, although we briefly discuss some mechanisms of **post-mutation evolvability [G]** , where recombination plays an especially important role[13].

[H1] Phenotypic heterogeneity

Heritable phenotypic variation is the raw material of natural selection, and the best-known mechanisms to create such variation are DNA mutation and recombination. However, because the role these mechanisms play in generating phenotypic variation is well established and has been extensively reviewed[13,14], we here focus on another class of mechanisms whose astonishing diversity is only beginning to come to light through recent experimental work[15]. These mechanisms create phenotypic heterogeneity *without* creating genetic variation.

Non-genetic mechanisms to create phenotypic heterogeneity can be found in many processes affecting the expression of genetic information. We review four such mechanisms: stochastic gene expression, errors in protein synthesis, epigenetic modifications, and protein promiscuity. Each mechanism can create phenotypic variation in a population of genetically identical individuals[16]. Such variation can for example provide a competitive advantage to subpopulations with adaptive phenotypes in fluctuating environments[17,18]. These phenotypes may themselves be heritable, eventually made permanent by mutation or epigenetic modification, or they may simply 'buy time' for a population to adapt in other ways to an environmental challenge (Fig. 1a).

[H2] Stochastic gene expression. Stochastic gene expression, or **gene expression noise [G]** has multiple causes, including the efficiency of transcription and translation[19,20], as well as the regulation of gene expression by low-abundance molecules whose numbers fluctuate randomly in a cell[21] (Fig. 1b). It can create non-genetic, adaptive diversity in phenotypes as diverse as **viral latency [G]** , bacterial **competence [G]** and antibiotic resistance, as well as drug resistance in cancer[22-24].

One example where stochastic gene expression causes adaptive phenotypic variation is persistence, where some cells in an isogenic population exhibit a physiologically dormant phenotype called a persister phenotype[25]. This phenotype is adaptive, because a dormant subpopulation has the potential to survive drugs that require active growth for killing, affording the persistent subpopulation time to acquire resistance-conferring DNA mutations. This was recently demonstrated in a laboratory evolution experiment of *Escherichia coli* populations subjected to intermittent exposures of ampicillin[26], in which persistence served as a stopgap until some individuals acquired resistance-causing mutations.

Persistence arises in only a small fraction of a population, so one might think that the resulting **population bottleneck [G]** would hinder evolvability by reducing the supply of beneficial mutations. However, a recent study of non-small-cell lung cancer indicates that this need not be the case[27]. These cells stochastically express a persistent phenotype, mediated by an altered chromatin state[28]. A population derived from one of these cells was exposed to the drug erlotinib, which resulted in the formation of multiple persistent subpopulations. Seventeen of these subpopulations were later expanded in isolation from each other until drug resistance emerged through DNA mutations. Genetic analysis of the resistant clones uncovered several distinct resistance mechanisms, indicating that several evolutionary paths to resistance remained despite the population bottleneck. In sum, persistence can facilitate evolvability, because it allows some individuals (individual cells in this example) to survive long enough to experience adaptive genetic change.

Rare-cell-variability is similar to persistence, in that a subpopulation of cells stochastically expresses a phenotype that facilitates the evasion of drug treatment[28,29]. It is different from persistence, in that the subpopulation is not dormant, but rather exhibits a transient transcriptional state that may include the expression of resistance-conferring genes. For example, in a study of resistance evolution to the drug vemurafenib in human melanoma, rare cells transiently expressed one or several such genes prior to drug exposure, making them 'pre-resistant'.[24] After four weeks of drug exposure, stably resistant colonies emerged that expressed these genes at uniformly high levels, and in a semi-coordinated fashion. For instance, of 1,456 genes known to contribute to resistance, pre-resistant cells expressed 72. After four weeks of drug exposure, this number rose to 966. These changes were not caused by DNA mutations. Rather, drug exposure initiated epigenetic cellular changes that stabilized the transiently resistant state. The transient expression of resistance-conferring genes in rare cells is not limited to melanoma, but is also found in unrelated cancer cell types, suggesting that the epigenetic conversion of a rare, transient transcriptional state to a stably resistant state may be a common mechanism of evolvability in cancer[30]. Such stabilization of a new phenotype, even if temporary, may facilitate more permanent stabilization through genetic mutations. Examples like these are closely related to the phenomenon of **genetic assimilation [G]** , which has been studied since the 1950s[31,32].

Stochastic gene expression may also facilitate evolvability by changing how strongly mutations affect fitness, and in particular by enhancing the positive effects of beneficial mutations[33]. This was recently demonstrated using synthetic gene circuits in *Saccharomyces cerevisiae*[34], which were engineered to exhibit varying degrees of expression heterogeneity in an antifungal resistance gene. Populations harbouring a version of a circuit with high expression heterogeneity were compared to those harbouring a circuit with low expression heterogeneity. During an evolution experiment where populations were exposed to increasing concentrations of the antifungal drug fluconazole, high-heterogeneity populations went extinct less often and evolved higher fluconazole resistance than low-heterogeneity populations. At least partly responsible were the increased beneficial effects of flucanozole resistance mutations in high-heterogeneity populations, because the same

130 resistance mutations conferred greater resistance when expressed with high expression heterogeneity than
131 with low heterogeneity. Altering the phenotypic effects of mutations is therefore another route by which
132 stochastic gene expression can facilitate evolvability[33].

133

134 ***[H2] Errors in protein synthesis.*** In addition to stochastic gene expression, protein synthesis errors can also
135 create non-genetic phenotypic heterogeneity. Such errors come in many forms and occur at multiple stages of
136 protein synthesis, including nucleotide misincorporation during transcription, tRNA misacylation during
137 translation, and **kinetic trapping [G]** during protein folding[35]. Translation is particularly error-prone, with
138 rates of mistranslation exceeding those of DNA point mutations by several orders of magnitude. Such errors
139 are also called phenotypic mutations[36], and they include missense, read-through, and frameshift mutations.
140 Phenotypic mutations can facilitate evolvability, because they create variation in a protein pool expressed
141 from the same gene, and some of this variation may be adaptive (Fig. 1c). For example, elevated
142 mistranslation rates in *Mycobacterium tuberculosis* generate variation in the beta subunit of RNA
143 polymerase, which increases resistance to the antibiotic rifampicin[37]. Similarly, mistranslation of CUG
144 codons in the fungal pathogen *Candida albicans* generates variation in cell surface proteins that facilitate
145 evasion of the host's immune system[38].

146

147 A special kind of mistranslation error is **stop-codon readthrough [G]** , which is a common mechanism for
148 generating protein variation in species as different as yeast, fly and human[39,40]. In fungi, for example, it can
149 lead to the expression of cryptic peroxisomal signalling motifs that create variation in the cellular localization
150 of proteins[40]. In crustacea and hexapods, DNA sequences downstream of an affected stop codon are often
151 evolutionarily conserved, suggesting that stop-codon readthrough occurs frequently enough to affect the
152 evolution of cryptic sequences[41,42].

153

Protein synthesis errors not only enhance evolvability by increasing protein diversity, they can also help pave the way for subsequent adaptive genetic change[43,44]. An example comes from the *S. cerevisiae* protein *IDP3*, an NADP-dependent isocitrate dehydrogenase that localizes to the peroxisome[45]. The protein originated in an ancient yeast whole-genome duplication, and diverged from its cytosolic ancestor *IDP2* by acquiring a C-terminal peroxisomal targeting signal, while *IDP2* remained cytosolic. Yeast species that diverged before the whole-genome duplication possess only a cytosolic *IDP2* gene, but in four of these species the gene contains a cryptic peroxisomal targeting signal in the 3′ untranslated region. This signal can be revealed via a +1 translational frameshift that bypasses the stop codon, which exposes the mistranslated protein to selection for peroxisomal targeting and function, and can, for example, lead to an increase in the strength of the peroxisomal signalling motif[45]. The frameshift is induced by a sequence context that is prone to ribosomal slippage, and that is also prone to single nucleotide deletions that mimic the effect of the frameshift on protein sequence. This correlation between phenotypic and genotypic mutations thus facilitated the evolution *IDP3*: Before the whole-genome duplication, *IDP2* could already be expressed in two locations: in the cytosol through faithful translation, and in the peroxisome through mistranslation. After the whole-genome duplication, the peroxisomal localization and function was made permanent via a single base deletion in one of the gene copies.

[H2] Epigenetic modifications. Phenotypic heterogeneity can also be caused by epigenetic changes, such as methylation of DNA and histones, alteration of chromatin structure, and the changes in protein conformation known as **prions [G].** For example, the prion [*PSI*$^{+}$] in *S. cerevisiae* is an aggregated conformation of the translational suppressor protein Sup35, which can be inherited by forming inactive complexes that convert other Sup35 proteins to the same inactive state[18]. Such aggregation reduces translational fidelity, which causes translational errors that include stop-codon readthrough events and frameshifts in other proteins[46] (Fig. 1d). Some of these errors reveal **cryptic genetic variation [G]** , producing phenotypes that are heritable and that can be adaptive[18,47]. For example, [*PSI*$^{+}$] can improve growth on a variety of carbon and nitrogen sources, and in various temperatures and stress conditions[18,48]. The phenotypes induced by [*PSI*$^{+}$] and other prions can

persist for generations, which provides opportunity for the phenotypes to be reinforced by mutation or recombination, or to interact with existing genetic variation or new mutations to form novel, potentially adaptive phenotypes[47,49]. Recent research in this area has greatly expanded the repertoire of known prions[49-51], elucidated the mechanisms by which they confer a selective advantage[52-54], and uncovered alternative forms of protein-based inheritance[55-57]. For instance, the first bacterial prion has recently been identified[50]. It is the transcription terminator Rho of *Clostridium botulinum*, which can take on one of two conformations, a soluble form that does not impact transcription, and an aggregate prion form that can self-propagate and that alters transcription, causing genome-wide transcriptomic changes. Its discovery raises the exciting possibility that this cause of evolvability is ancient and predates the origin of eukaryotes.

The methylation of DNA and histones are heritable epigenetic modifications, which create phenotypic variation that can be adaptive[58,59]. A recent example comes from the study of intra-tumour heterogeneity in cancer[60]. Proliferative potential varies among cancer cells within the same tumour, and those cells that preserve proliferative potential can drive long-term tumour growth. Some of this variation is caused by an epigenetic modification to an **enhancer [G]** that modulates the expression of the linker histone H1.0, which is involved in the compaction of chromatin. Specifically, DNA methylation of the enhancer represses the expression of the linker histone. This destabilizes nucleosome–DNA interactions, which de-represses the expression of oncogenes that support proliferative potential. Thus, variation in the epigenetic modification of a regulatory element creates variation in chromatin structure, some of which facilitates cancer cell self-renewal. This epigenetic cause of intra-tumour heterogeneity is found in dozens of cancers[60], and it is just one of several epigenetic causes of phenotypic heterogeneity in this disease[59].

[H2] Protein promiscuity. A fourth cause of evolvability-enhancing phenotypic heterogeneity is protein promiscuity[61,62]. Promiscuous proteins have one primary adaptive function and other secondary latent functions. Prominent examples include enzymes with 'moonlighting' catalytic activities[63,64], such as bacterial

carbonic anhydrase II, which mainly catalyzes the reversible hydration of carbon dioxide, but also exhibits promiscuous activity toward esters[61]. Promiscuity can facilitate evolvability, because it provides a reservoir of potentially adaptive protein activities that can be enhanced by gene duplication, when such duplications are followed by mutations that refine different activities in different duplicates. For example, in *S. cerevisiae*, two transcription factors that are products of a past gene duplication regulate the genes involved in maltose metabolism and the genes involved in palatinose metabolism[65]. These duplicates arose from a single promiscuous transcription factor that regulated the expression of both the maltose- and palatinose-specific genes. After gene duplication, two single-nucleotide mutations in the DNA binding domain of one of the duplicates altered its binding specificity, such that it could no longer bind the promoters of the maltose-specific genes. Mutations in the coding region of the other duplicate weakened its activity toward maltose, such that it could only activate the maltose-specific genes, because their promoters contained multiple binding sites for the protein, which compensated for its reduced activity. Gene duplication thus facilitated the partitioning of the promiscuous activity of a single transcription factor among its duplicates.

Sometimes duplication may not even be needed to reinforce a promiscuous function[66,67]. This is especially true for regulatory elements. For example, the *Drosophila santomea* gene *Neprilysin-1* evolved a novel expression pattern in the fly's optic lobe via a small number of mutations to an existing enhancer[68]. Reconstruction of the enhancer's ancestral state revealed its promiscuous activity in the optic lobe, indicating that these mutations did not generate new enhancer activity *de novo*, but rather refined one of the enhancer's existing, latent activities.

In sum, these examples show how various forms of phenotypic heterogeneity — caused by stochastic gene expression, errors in protein synthesis, epigenetic modifications, and protein promiscuity — facilitate the exploration of novel phenotypes. Some of these phenotypes may be adaptive, and may be made permanent by selection for genetic or epigenetic changes that reinforce the phenotype. We emphasize that many other

mechanisms to regulate molecular processes exist, and given the adaptive benefits of phenotypic heterogeneity, it is likely that they will also be implicated in producing such heterogeneity.

[H1] Robustness

Robustness to DNA mutations can be viewed as a dual, converse, or opposite property to non-genetic phenotypic heterogeneity. Whereas non-genetic phenotypic heterogeneity implies that phenotypic variation exists in the absence of genetic variation, robustness implies that phenotypic variation does *not* exist in the *presence* of genetic variation, because a phenotype is robust to genetic change.

Many phenotypes are to some extent robust to mutations[69,70]. Examples include the structure and biological activity of macromolecules[71], the gene expression patterns of regulatory networks[72], and the ability of a metabolism to synthesize biomass[73]. Such robustness can also be enhanced in various ways. For example, DNA mutations that enhance protein stability can also enhance robustness, because enhanced protein stability increases the range of mutations a protein can experience while still folding into its native structure[71]. Gene duplication can also enhance robustness, because it causes gene functions to become redundant, and can thus increase the incidence of mutations that can be tolerated by either duplicate[74] (but see refs [75,76]). **Chaperones [G]** such as the eukaryotic protein Hsp90 enhance robustness in organisms as diverse as fruit flies, cave fish, plants and bacteria[77-82], although such buffering may not occur in all organisms and may not affect all genetic variation[78,83].

In each of these cases, DNA mutations can cause genetic diversity without changing a phenotype. Such cryptic genetic variation can facilitate evolvability in at least three ways. First, cryptic genetic variation may be revealed as phenotypic variation, for example via the partial loss of function of a chaperone or via the appearance of a prion, or when the environment changes[18,42,47,78,81,84,85]. Because these phenotypes are occasionally exposed to selection, cryptic genetic variation may be enriched for adaptations[42]. Second, cryptic

genetic variation provides many distinct genetic backgrounds in which the effects of new mutations can manifest themselves[86,87]. This can be advantageous because the same mutation can have different phenotypic effects — neutral, beneficial, or detrimental — in different genetic backgrounds, a phenomenon caused by frequent **epistatic interactions [G]** (non-additive interactions) among mutations. Finally, cryptic genetic variation may give rise to new phenotypic variation via recombination.

The study of robustness has a long history in evolvability research[69,88], but recent experimental work has greatly expanded our mechanistic understanding of how robustness facilitates the generation of adaptive phenotypic variation. These advances largely result from technological progress in areas such as deep mutational scanning and ancestral protein reconstruction (Box 1). We highlight recent examples from individual macromolecules, from interactions between macromolecules and their ligands, and from entire gene regulatory networks.

The C2H2 zinc finger is the most prominent **protein domain [G]** in many metazoans, but not in other eukaryotes. It occurs in C2H2 zinc finger transcription factors, where multiple copies of this domain are typically arranged in tandem, such that each domain contacts three or more DNA bases, the identity of which is determined by four base-contacting amino acids in the domain's alpha helix. The diversity of DNA sequences recognized by metazoan C2H2 zinc fingers far exceeds that of other eukaryotic C2H2 zinc fingers, and recent research implicates robustness in their expansion and diversification[89]. Specifically, in metazoans, non-base-contacting amino acids of the C2H2 zinc finger domain form hydrogen bonds with the DNA phosphate backbone to enhance binding energy. By contrast, the binding energy of other eukaryotic C2H2 zinc fingers depends primarily on base-contacting amino acids. This suggests that the non-base-contacting amino acids of metazoan C2H2 zinc fingers confer robustness of DNA binding to mutations in base-contacting amino acids, which facilitates the diversification of DNA binding preferences.

The evolution of steroid receptor binding preferences provides another example of how robustness facilitates evolvability. Steroid receptors are transcription factors that can be classified according to their binding preference for oestrogen response elements or steroid response elements. These two response elements are 6nt-long DNA sequences that differ by just two nucleotides. The ancestral steroid receptor from which all steroid receptors descended more than 450 million years ago binds oestrogen response elements[90]. After this protein duplicated, one daughter protein retained specificity to oestrogen elements, whereas the other evolved a preference for steroid response elements. This shift in specificity required eleven substitutions outside of the DNA binding domain and three substitutions within it. The eleven mutations outside of the DNA binding domain did not affect DNA binding specificity — specificity was robust to genetic changes — but they had another important consequence: they dramatically altered the number of mutational variants capable of binding steroid response elements. Specifically, out of 160,000 possible mutational variants of the ancestral protein *without* the 11 mutations, only 41 specifically bound steroid response elements. By contrast, of the same 160,000 mutational variants of the ancestral protein *with* the 11 mutations, 829 specifically bound steroid response elements, and these variants were accessible via fewer mutations[91]. The mutational neighbourhoods of the two proteins were therefore dramatically different, and it was the robustness to mutation that facilitated access to the mutational neighbourhood that conferred higher evolvability (Fig. 2).

Not only are regulatory proteins robust to mutation, so too are the regulatory elements they target[87,92]. For example, eukaryotic transcription factors typically bind dozens to hundreds of distinct nucleic acid sequences[93], which tend to be mutationally interconnected, such that a mutation to a sequence that binds a transcription factor will often generate another sequence that also binds the transcription factor[87]. This robustness facilitates the accumulation of genetic diversity in binding sites[94], which provides distinct genetic backgrounds in which to test new mutations. Some of these mutations generate binding sites for other transcription factors[87], which may lead to adaptive gene expression changes.

Gene expression patterns themselves are highly robust, not only to mutations in binding sites, but also to wholesale changes in the number, identity, and orientation of binding sites within regulatory regions[95], and thus to changes in the structure of gene regulatory networks[96]. Modelling work has long anticipated that such robustness can facilitate evolvability[97,98], but empirical support for this possibility was only recently provided[99]. Specifically, the highly conserved fungal transcription factor *Ndt80* underwent a pronounced switch in function from an ancestral role regulating meiosis and sporulation to a derived role regulating biofilm formation. Experiments with six different extant yeast species suggest that this shift was not caused by a change in the binding specificity of *Ndt80*, but rather by gains and losses of binding sites for *Ndt80*. These changes preserved the ancestral role of *Ndt80* but allowed the regulatory network controlling meiosis and sporulation to sample many architectural configurations. This sampling facilitated the discovery of a network configuration that supported the derived role of biofilm production in *Candida albicans*.

In sum, these examples illustrate that robustness creates opportunities for the exploration of novel genotypes, some of which constitute or lead to new adaptations. Other pertinent examples include recent studies of robustness in viral proteins[100,101], bacterial enzymes[102], tumour suppressor genes[103], protein–protein interactions[104,105] and gene regulatory networks[106].

[H1] Adaptive landscape topography

An adaptive landscape is an analogy to a physical landscape, in which each location or coordinate in a physical space corresponds to a genotype in an abstract **genotype space [G]** [107], and where the elevation at this location corresponds to the fitness of this genotype[108]. One can view adaptive evolution as a process where populations of ever-changing genotypes explore such a landscape through random DNA mutations and recombination, and where natural selection helps such populations discover peaks or plateaus of high fitness. Adaptive landscapes are central to evolvability research, because the topography of an adaptive landscape, and a population's location within a landscape, determine the amount of beneficial phenotypic variation that

mutations can create. A smooth, single-peaked landscape facilitates evolvability, because mutation can bring forth beneficial phenotypic variation from anywhere in the landscape, except atop a global peak (Fig. 3a). In contrast, a rugged landscape can hinder evolvability, because the local peaks it contains may attract an evolving population and preclude the generation of further beneficial phenotypic variation (Fig. 3b). Moreover, the shape of an adaptive peak — **concave [G]** versus **convex [G]** — affects the amount of beneficial phenotypic variation that mutation can bring forth as an evolving population ascends the peak. Until recently, most work on adaptive landscapes was theoretical, but experiments are now being increasingly used to characterize the topography of adaptive landscapes[109]. Some of these studies use organismal fitness to define the surface of a landscape[110,111], whereas others use molecular phenotypes, such as the enzymatic activity[112,113] or binding affinity[114,115] of a protein, and are therefore also referred to as genotype–phenotype landscapes[116]. The pace of this work is still accelerating, and we focus on the most recent such work.

Perhaps the most important factor affecting landscape ruggedness and the shape of adaptive peaks is epistasis — non-additive interactions among two or more mutations[117,118]. Epistasis can take different forms (Fig. 3c,d), and can occur with mutations that are individually deleterious or beneficial. For example, negative epistasis amongst beneficial mutations occurs when the combined effect of the mutations is smaller than the sum of the individual mutational effects[119,120] (Fig. 3c). It is also referred to as antagonistic or diminishing returns epistasis. Positive epistasis amongst beneficial mutations occurs when the combined effect of the mutations is larger than the sum of the individual mutational effects (Fig. 3c). It is also referred to as synergistic epistasis. The terminology used to describe epistasis can be confusing (e.g., synergistic epistasis is also used to describe negative epistasis amongst deleterious mutations[121]), but mathematically the definition of positive and negative epistasis is straightforward. Epistasis amongst two mutations A and B can be quantified as $\varepsilon = f_{ab} + f_{AB} - f_{Ab} - f_{aB}$, where f is the phenotype or fitness of the 'wild type', double mutant, and single mutant genotypes, respectively. Negative epistasis occurs when $\varepsilon < 0$, whereas positive epistasis occurs when $\varepsilon > 0$.

Another important form of epistasis is sign epistasis[122]. It occurs when the sign — beneficial (+) or detrimental (–) — of a double mutation differs from that of one or both of the constituent single mutations. For example, whereas both single mutations may be individually detrimental, they may be jointly beneficial. Sign epistasis creates local valleys or peaks and thus ruggedness in an adaptive landscape (Fig. 3d)[118]. In doing so, it can affect the amount of adaptive variation accessible to a population, a population's evolutionary trajectory, and its ability to reach a global peak. For example, global peaks may be inaccessible if all evolutionary trajectories to them require traversing one or more adaptive valleys, which is disfavoured by natural selection and possible only under restricted conditions[123,124]. With some exceptions[125-127], sign epistasis thus reduces evolvability.

A fundamental challenge in mapping an adaptive landscape is that the number of genotypes in a typical genotype space is so vast that their phenotype or fitness cannot usually be exhaustively measured. One approach to overcome this challenge uses experimental evolution of whole organisms[128], where the change in a population's mean fitness and genotypic composition is monitored while the population evolves for hundreds or thousands of generations in the laboratory. Such experiments show that even though specific genetic changes that cause fitness increases are usually not predictable, the evolutionary trajectory of mean fitness increases can be highly predictable[129-132], suggesting that suitable statistical methods may be able to infer general statistical properties of adaptive landscape topography[133,134]. Additionally, experimental evolution demonstrates that a population's mean fitness increase — a proxy for evolvability — depends primarily upon the fitness of the starting genotype, and also upon the starting genotype itself (i.e., from which location a population begins to explore an adaptive landscape)[129,135].

An important limitation of this method is that it does not allow the detailed mapping of adaptive landscape topography, because evolving populations typically harbour a large number of mutations whose contributions to fitness are not easily disentangled[136,137]. Such a mapping requires more targeted approaches. One such

approach is to engineer all possible genotypes in a small region of a landscape, for example by using all combinations of the presence or absence of mutations that occurred along an adaptive evolutionary pathway, or more comprehensively by using all possible combinations of mutations at a fixed number of nucleotide or amino acid sites[109] (Fig. 3e). One pertinent recent study constructed an adaptive landscape from all possible combinations of 13 amino-acid-changing mutations at six amino acids in the heat-shock protein Hsp90 of *S. cerevisiae* in a high-salt environment[138]. The resulting landscape provides several fundamental insights into the evolvability of Hsp90 in this challenging environment. First, the landscape is dominated by epistasis: not a single pairwise interaction between mutations is additive. These epistatic interactions include both positive and negative epistasis, as well as sign epistasis. Second, the sign epistatic interactions produce landscape ruggedness, with five local peaks and a single global peak that conveys a 10% increase in yeast growth rate on high salt, relative to the wild-type genotype. Third, although the landscape is moderately rugged, it is still highly navigable, as shown by simulated **adaptive walks [G].** These walks reveal that the global peak can be reached from nearly any starting point in the landscape. One important exception is the wild-type genotype, because adaptive walks starting from this genotype tend to converge to a local peak but not to the global peak. Taken together, these observations show how epistasis can generate landscape ruggedness, and that a population's location within such a rugged landscape affects the ability of mutation to bring forth heritable, adaptive phenotypic variation.

Another approach to constructing adaptive landscapes is based on deep mutational scanning[139], in which phenotypes are assayed for a large number of mutational variants of a single, typically wild-type genotype (Fig. 3f). This approach thus characterizes the immediate neighbourhood of an adaptive peak. It has been used extensively in recent years, for phenotypes as different as the 'splicing-in' of an exon[116], the binding affinity[114,115] and enzymatic activity[112,113] of a protein, as well as the fitness of an entire organism[84,110,111]. For example, a recent study employed a deep mutational scan of the wild-type sequence of the green fluorescent protein from the jellyfish *Aequorea victoria*, using fluorescence level to define the landscape's surface[140]. This analysis revealed a single, narrow peak centred on the wild-type sequence, with three quarters of the

single-mutant sequences displaying reduced fluorescence, and half of the sequences with four mutations showing no fluorescence at all. The analysis also revealed abundant negative epistasis, and very little positive epistasis. Negative epistasis produces concave peaks[141] (Fig. 3c), which reduces evolvability when a population approaches an adaptive peak, because the amount of adaptive phenotypic variation accessible via mutation decreases. Conversely, positive epistasis helps create convex peaks and facilitates evolvability. These modes of epistasis also have implications for mutational robustness[141,142]. The concave peaks formed by negative epistasis confer robustness, because individual mutations to genotypes on such peaks have small fitness effects. By contrast, the convex peaks formed by positive epistasis confer sensitivity to mutation, because individual mutations to genotypes on such peaks have large fitness effects. With few exceptions[143,144], a bias towards negative epistasis is among the most commonly reported features of experimentally characterized adaptive landscapes[110,111,114,115,138,140,141], in agreement with the diminishing returns epistasis regularly observed in laboratory evolution experiments[119,120,130-132].

Even though deep-mutational scanning and related techniques are powerful, they still render a typical genotype space sparsely sampled, and extrapolating insights from the resulting incomplete landscapes to complete landscapes is challenging[138,145,146]. Not affected by this limitation are small genotype spaces, where it is possible to assay the phenotypes of all possible genotypes[147,148] (Fig. 3g). One such genotype space is that of short transcription factor binding sites, where one can measure how strongly a transcription factor binds to thousands of different DNA sequences[93]. Such information is not just available for one, but for thousands of transcription factors from multiple species[149]. Binding strength is an important molecular phenotype, because it is a proxy for a factor's ability to activate or repress a target gene, and the gene expression patterns that emerge from such binding events embody fundamental biological processes, including those in development, physiology, and behaviour. Importantly, the location and timing of these gene expression patterns can be fine-tuned, or altogether transformed, by mutations that affect the strength of transcription factor–DNA interactions[150,151]. The mapping of DNA sequence to binding strength can therefore be thought of as an

adaptive landscape, in which mutation and natural selection optimize the capacity of a DNA sequence to bind a transcription factor.

A recent study analyzed the topographies of more than 1000 such landscapes[94]. They contained little sign epistasis, and therefore typically comprised only a single peak. Similar to the landscape of yeast Hsp90 in high salinity[138], these landscapes were highly navigable. Their global peaks tended to be accessible from throughout the landscape via a series of 'uphill' mutational steps. Indeed, even at the furthest mutational distance from a global peak, more than 20% of all possible mutational paths were accessible. Such smooth landscapes facilitate evolvability, because mutation can readily bring forth beneficial phenotypic variation, regardless of a population's location on the landscape.

A limitation to these approaches, as compared to experimental evolution, is that an adaptive landscape for a single binding site or an individual gene has many fewer dimensions than an adaptive landscape for an entire genome. This is important, because the valleys that separate adaptive peaks in low-dimensional landscapes may not do so in high-dimensional landscapes. The reason is that increased dimensionality may create mutational paths that bridge adaptive valleys, or that transform local adaptive peaks into **saddle points [G]**. Such **extra-dimensional bypasses [G]** increase the accessibility of adaptive peaks, and thus increase evolvability[5]. Long the subject of theoretical research[5,152], extra-dimensional bypasses have recently been uncovered in an adaptive landscape of binding affinity for the protein GB1 of Streptococcal bacteria[153]. The authors analyzed all 20^4 protein variants of 4 amino acid sites, and sampled ~20,000 pairs of mutations that exhibited reciprocal sign epistasis (Fig. 3d). Of these pairs, ~15% exhibited an extra-dimensional bypass when one of the other two amino acid sites was considered. Such an increase in the mutational accessibility of adaptive peaks suggests that increasing the dimensionality of adaptive landscapes from that of individual binding sites or genes to that of entire genomes reduces landscape ruggedness and thus enhances evolvability.

457 The examples highlighted here are only a small sample of recent experimental studies of adaptive landscapes,
458 with other pertinent examples in systems as different as drug delivery vehicles[154] and cancer[155]. We anticipate
459 that the resolution and scale of such landscapes will continue to increase as high-throughput genotyping and
460 phenotyping technologies advance (Box 1).
461
462 **[H1] Evolvability evolving**
463 Any cause or mechanism of evolvability could in principle itself be subject to evolutionary change. Three
464 questions about such change are germane. First, can the mechanism evolve in principle, i.e., is there genetic
465 variation in it? Second, does it evolve, either in nature or in the laboratory? Third, is a change in evolvability
466 itself adaptive? Or is it instead a by-product of other adaptations or of non-adaptive processes, such as
467 developmental constraints, mutation bias, or genetic drift? We discuss existing evidence pertaining to these
468 questions for each of our three major causes of evolvability.
469
470 ***[H2] Evolution of phenotypic heterogeneity.*** Genetic mechanisms that create phenotypic heterogeneity can
471 evolve. For example, the rate of DNA mutation is itself subject to evolutionary change[156,157], because the
472 DNA repair enzymes that keep DNA mutations in check can themselves undergo mutations that lead to
473 elevated mutation rates. Such evolution can be adaptive in novel environments[156,158], for example during
474 *E.coli*'s colonization of the mouse gut[159]. Similarly, increases in recombination rate can accelerate a
475 population's rate of adaptation — a proxy for evolvability — either by creating more beneficial allele
476 combinations or by helping to eliminate deleterious mutations[160].
477
478 Non-genetic mechanisms of phenotypic heterogeneity can also evolve[161]. For example, gene expression noise
479 levels vary genetically with promoter strength and with the strength of transcription factor binding sites[162];
480 stop-codon readthrough rates vary with stop-codon identity (UAG, UAA or UGA), the surrounding sequence
481 context, and the structure of mRNA[163]; the formation and activity of prions varies according to the presence
482 of aggregation-prone amino acid sequences in prion-forming protein domains, such as glutamine/asparagine-

483 rich sequences[164]; and protein promiscuity varies with a protein's coding sequence[61,67,105]. Thus, in each case,
484 the factors that can affect phenotypic heterogeneity are genetically encoded, and can therefore evolve.
485
486 What is more, mechanisms that create phenotypic heterogeneity do evolve, both in laboratory experiments
487 and in nature. For example, the evolution of increased gene expression noise in *S. cerevisiae* has been
488 reported for antifungal resistance genes in the lab[34] and for plasma-membrane transporters in the wild[165].
489 Experimental evolution of synthetic *E. coli* promoters to specific mean expression levels results in promoters
490 with low expression noise, suggesting that the noisy expression of many natural *E. coli* promoters is an
491 evolved property[166]. Other forms of phenotypic heterogeneity have also been successfully evolved in the lab,
492 including protein promiscuity in bacteriophage λ (ref [67]) and the stochastic switching of colony morphology
493 in *Pseudomonas fluorescens*[17].
494
495 At least in some instances, the evolvability conferred by phenotypic heterogeneity may have evolved because
496 it was adaptive. For example, in the experimental evolution of populations of *S. cerevisiae* exposed to
497 antifungal stress, increased expression noise evolved in the synthetic regulatory circuits controlling an
498 antifungal resistance gene, because it enhanced the adaptive value of beneficial mutations[34]. Similarly, in the
499 experimental evolution of populations of *P. fluorescens* exposed to environmental fluctuations, the stochastic
500 switching of colony morphology evolved as an adaptive bet-hedging strategy[17]. Such a strategy was also
501 observed in the experimental evolution of *E. coli* under antibiotic stress, where the stochastic expression of
502 persister cells evolved to facilitate survival in high concentration of antibiotic[26]. In other instances,
503 evolvability is a by-product of other adaptations. For example, promiscuity in the host-recognition protein of
504 bacteriophage λ evolved as a by-product of selection for increased absorption to the virus' native cell surface
505 receptor[67]. Specifically, the same mutations that increased absorption also destabilized the protein, producing
506 λ particles that were proficient at targeting different receptors.
507

[H2] Evolution of robustness. Variation in mutational robustness is found at all scales of biological organization, including the structures of macromolecules[71,147], interactions between macromolecules and their ligands[87,92], as well as the gene expression patterns of regulatory circuits[167]. Mutational robustness can therefore evolve. Moreover, it can evolve by various means; for example, via increased protein stability[71] or via gene duplication[74].

Mutational robustness also has evolved, both in nature and in the laboratory. For example, the structures of eukaryotic microRNA precursor stem-loops are more robust to mutation than random RNA sequences with similar stem-loop structures[168], and the mutational robustness of a protein's tertiary structure tends to increase with the protein's age[169]. Directed protein evolution has demonstrated that mutational robustness of cytochrome P450 proteins can increase in sufficiently large populations[170], and experimental evolution of *S. cerevisiae* has demonstrated that gene duplications can confer mutational robustness[74].

We are not aware of experimental evidence that mutational robustness has evolved because it causes evolvability. By contrast, there is evidence that mutational robustness has evolved because it is itself adaptive[171], for example in viral populations exposed to chemical mutagens, because robustness provides a competitive advantage when the mutation rate is elevated[172]. In addition, mutational robustness may often evolve as a by-product of other adaptations. For example, chaperones help maintain proteome integrity during environmental stress, and may buffer mutations only as a side effect. Similarly, the mutational robustness of eukaryotic microRNA precursor stem-loops is likely to be a by-product of selection for robustness of these RNA structures to temperature fluctuations[173].

[H2] Evolution of adaptive landscape topography. This cause of evolvability can also evolve: the location of an individual or a population on an adaptive landscape can change through DNA mutations or recombination, and because local landscape topography may differ in different locations, so may evolvability[91,135,138,141,147,174-176]. A comparison of the fitness effects of mutations to three orthologous TIM barrel proteins provides an

illustrative example[175]. These proteins are distantly related, retaining only ~30–40% sequence identity, but they have the same fold and function. They therefore occupy different locations on the same adaptive landscape. These locations differ in their evolvability, because the same mutations have different, albeit correlated fitness effects in the three sequence backgrounds (locations). Another example is provided by the experimental evolution of two divergent yeast strains in the same laboratory conditions[129]. These strains, which differ at roughly 50,000 single nucleotide sites and therefore occupy different locations on their adaptive landscape, also differ in the rate at which they adapt evolutionarily — a proxy for evolvability[129,177]. Analysis of **quantitative trait loci** **[G]** partly attributes this difference in evolvability to a small subset of mutations, such as those involved in the ribosome biogenesis pathway.

The evolvability conferred by a landscape's local topography has also evolved. As shown in Fig. 2, for example, eleven substitutions occurred during the evolution of an ancient steroid hormone receptor, and this change in adaptive landscape location dramatically altered the spectrum of DNA-binding phenotypes accessible via mutation[91]. An additional example comes from Lenski's long-term (>60,000 generations) evolution experiment with *E. coli* populations[178]. Here, one out of twelve populations evolved the ability to utilize citrate, and did so after 31,500 generations. The mutation needed to evolve citrate utilization conferred a fitness benefit even in the original ancestor of the experiment, but other mutations that occurred during the initial stages of the experiment conferred larger fitness benefits, and created a genetic background in which the initial citrate utilization-mutation no longer conferred a fitness benefit. Thus, evolution drove the population to a location on the adaptive landscape that precluded the evolution of citrate utilization. Only later did subsequent mutations bring the population back to a location where this mutation was adaptive.

The same experiment also provides further evidence for evolving evolvability[177]. Within the first 500 generations of this experiment, multiple genetically distinct subpopulations had evolved within a single population, meaning that the population had diversified from the location of the ancestral genotype to multiple new locations on the adaptive landscape. One of these subpopulations would eventually outcompete

560 the others, but it was not the subpopulation with the highest fitness. Rather, it was a subpopulation located in
561 a region of the adaptive landscape that had higher evolvability. This was shown by 'replay experiments', in
562 which 10 replicate populations were evolved from distinct founding subpopulations — that is, from distinct
563 locations on the adaptive landscape. The subpopulation that would eventually outcompete the others
564 generated more beneficial phenotypic variation than the other subpopulations — it had higher evolvability.
565 After ~900 generations of evolution from these distinct landscape locations, the subpopulations evolved from
566 the high-evolvability location tended to outcompete those evolved from other locations.
567
568 We are not aware of experimental evidence that a population's location on an adaptive landscape has evolved
569 because it conferred evolvability. For instance, in the preceding example, evolvability evolved as a by-
570 product of the fixation of neutral or beneficial mutations that just happened to drive one of the subpopulations
571 toward a high-evolvability region of the landscape[177]. Non-adaptive forces may also explain the evolution of
572 a population's location on an adaptive landscape. For example, the eleven substitutions that occurred during
573 the evolution of an ancient steroid hormone receptor did not alter the protein's binding specificity, which
574 suggests that genetic drift caused this change in landscape location and the corresponding dramatic shift in
575 evolvability[90]. An alternative possibility is that this change in landscape location was due to selection for
576 protein function unrelated to binding specificity.
577
578 Taken together, these examples show that the three causes of evolvability highlighted here — phenotypic
579 heterogeneity, robustness, and adaptive landscapes — are themselves subject to evolutionary change.
580 Whether they often evolve because they confer evolvability remains a particularly challenging open question.
581
582 **[H1] Outlook**
583 Driven by technological advances, research into all three causes of evolvability is progressing in leaps and
584 bounds. We anticipate that this progress is going to continue unabated. For example, the currently well-
585 studied mechanisms to create non-genetic phenotypic heterogeneity that we discuss may well be only a small

586 subset of all pertinent mechanisms. Future work may reveal others to be important as well, such as RNA
587 editing[179] and protein allostery[180]. In addition, we know little about how conflicts of selection may influence
588 the evolution of such mechanisms, especially in organisms that are not clonally related (Box 2). As for
589 robustness, we understand its causes well for some systems like proteins or duplicate genes, but much less
590 well for systems of greater complexity, such as gene regulatory circuits and metabolism. The evolutionary
591 consequences of robustness become amply clear from detailed reconstructions of the evolution of molecules
592 such as steroid hormone receptors[91], but to date few such reconstructions are available. In the context of
593 adaptive landscapes, we are only beginning to understand how landscape topography depends on higher-
594 order epistasis[181,182]. Moreover, although we know that the environment *can* affect adaptive landscape
595 topography, we know little about *how* it does[86,183]. We are also only beginning to understand how our
596 knowledge of landscape topography may facilitate the prediction of evolutionary trajectories[109,184], or the
597 deliberate redirection of evolving populations of pathogens toward low-evolvability regions of a landscape[185].
598

599 The three major causes of evolvability interact, but we do not fully understand how or to what effect. For
600 example, phenotypic heterogeneity can smoothen an adaptive landscape, if a genotype's overall fitness is
601 equal to the average fitness of each of the phenotypes it brings forth[33]. Similarly, a DNA mutation that
602 renders a protein's phenotype robust to further mutations can be viewed as displacing the genotype to a
603 smooth region of an adaptive landscape, where further mutations have smaller phenotypic effects. However,
604 the degree of such 'smoothing' has not been explicitly characterized for any experimentally studied
605 landscape. When an organism generates non-genetic adaptive variation in phenotypes, it creates two or more
606 phenotypes from the same genotype, but any one adaptive phenotype can be stabilized by DNA mutations
607 only if the starting genotype resides in a region of an adaptive landscape where some of its mutants provide
608 such stabilization. We do not know the extent to which non-genetic mechanisms that create phenotypic
609 variation and increase evolvability ensure that the variation they cause can be genetically stabilized. Finally,
610 because a phenotype's robustness to genetic and non-genetic change are often correlated[69], genotypes that are
611 especially robust to DNA mutations may also bring forth less phenotypic heterogeneity by non-genetic

means. If so, trade-offs between robustness and non-genetic mechanisms to create phenotypic heterogeneity may exist, and these trade-offs are well-worth exploring.

A final frontier regards the evolution of the various evolvability mechanisms themselves. As we have shown, there is ample evidence that all three mechanisms can and do change in biological evolution. However, we have less information about whether their existence reflects an adaptive value of evolvability. Does increased mutational robustness at least sometimes come about because it enhances evolvability? Has the ruggedness of some adaptive landscapes decreased in the course of evolution, and if so, is it because reduced ruggedness increases evolvability? Questions like these are fascinating and profound, because an affirmative answer means that life itself can help create the conditions that ensure its advancement.

Box 1 | **Methodological advances**

Our ability to study the molecular causes of evolvability has been greatly improved by recent methodological advances. For example, our growing understanding of phenotypic heterogeneity is driven by microfluidic devices and time-lapse microscopy, which provide information about the compositions, morphologies and growth rates of single cells in dynamic environments[186]. Complementary information is provided by methods such as fluorescence in situ hybridization and single-cell RNA-seq, which describe the location and abundance of mRNA transcripts, respectively[187,188]. Combined with whole-genome sequencing, such methods have detailed the molecular causes of phenotypic heterogeneity, such as how stochastic gene expression drives persistence in bacteria[26] and rare-cell variability in cancer[24]. Non-single-cell methodologies have also furthered our understanding of phenotypic heterogeneity. For example, ribosome footprint profiling, which characterizes the distribution of ribosomes on mRNA transcripts[189], has detailed the prevalence of stop-codon readthrough in yeast, fly, and human[39].

Several methodological advances have improved our understanding of mutational robustness and of adaptive landscapes. For example, approaches that characterize a small region of an adaptive landscape typically rely

638 on deep mutational scanning[139], a method that combines systematic mutagenesis with high-throughput
639 phenotypic assays. These assays include fluorescence-activated cell sorting, which can be used to measure
640 protein functions such as fluorescence or ligand binding, as well as EMPIRIC[190], which can measure the
641 fitness of many cells in parallel. To capture the effects of mutations in their native genomic context, genome-
642 editing tools such as CRISPR–Cas9 can be used to introduce mutations to specific chromosomal loci[103].
643 Approaches that exhaustively characterize an entire (small) genotype space have profited from chip-based
644 technologies that simultaneously assay the phenotypes of all possible genotypes[93], as well as from high-
645 throughput *in vitro* selection methodologies that systematically enrich an initially random library of
646 sequences for those sequences that perform a particular function, such as binding a ligand[147].
647 To understand how these causes of evolvability have changed over long evolutionary timescales, they are
648 often combined with maximum likelihood methods to statistically infer and experimentally reconstruct the
649 genotypes and phenotypes of ancient macromolecules[191].
650
651 Box 2 | **Conflicts between different levels of selection**
652 Biological systems are hierarchically organized, with macromolecules embedded in cells, cells in whole
653 organisms, and organisms in populations. A genetic change that is beneficial on one level of this hierarchy
654 may be detrimental on another. For example, because most random DNA mutations have detrimental effects
655 on individuals or their offspring[192], DNA mutations that increase the DNA mutation rate itself will also be
656 detrimental for most individuals. By contrast, they may be advantageous for a population as a whole,
657 especially in a stressful environment, where a few beneficial mutant individuals may ensure survival[158,193] or
658 accelerate adaptation[156]. Such conflicts are also relevant for the evolvability mechanisms we discuss, such as
659 those that generate non-genetic heterogeneity, because in most environments such heterogeneity will not
660 benefit all individuals[15,22,25]. Various approaches help predict how evolution can resolve such conflicts[194-198].
661 Among them are multi-level selection theory[197] and kin selection theory[196]. The latter shows that higher,
662 population-level adaptations can evolve and persist whenever populations consist of genetically highly
663 related individuals, because in this case, the genetic 'interests' of individuals are aligned with those of the

population. It is relevant here that many known cases of adaptive non-genetic heterogeneity are found in clonal populations of genetically identical individuals[15], where an individual's interests are served as long as some of its clone-mates survive. Although theoretical work shows that evolvability mediated by prions such as [*PSI*$^{+}$] may persist in non-clonal populations of the yeast *Saccharomyces cerevisae*[85,199], extending such insights to other mechanisms of phenotypic heterogeneity, particularly non-heritable mechanisms, and to a broader range of organisms remains an important task for future work.

With respect to robustness, the dual property to phenotypic heterogeneity, we note that it is often advantageous to an individual, for example when a mutation creates a thermodynamically more stable protein that is less prone to misfolding or inactivation[170]. Wherever this is the case, the individual-level advantage and the population-level advantage of evolvability are aligned. This makes robustness a cause of evolvability whose evolutionary origin need not involve conflict, and is thus especially easy to explain. At the same time, this absence of conflict also means that it is more difficult to disentangle whether the robustness of any one trait originated in an individual-level advantage, such as the robustness that chaperones provide to proteomes[200], or in a 'second-order' advantage of evolvability, which chaperones also provide[82].

Figure legends

Figure 1 | **Phenotypic heterogeneity is a cause of evolvability. a** | Phenotypic heterogeneity can generate a small subpopulation of cells that exhibits a new phenotype, such as a persister phenotype (red cells in environment 1). Such a phenotype can be adaptive, because it allows a subpopulation to survive an environmental challenge, such as antibiotic exposure (environment 2). Mutation (red cross) may stabilize the phenotype, or it may generate a different phenotype that is adaptive in the new environment, such as a mutation that confers resistance to an already tolerant bacterial cell. There are many sources of phenotypic heterogeneity: **b** | Stochastic gene expression causes mRNA transcript levels to vary among cells. **c** | Errors in protein synthesis, such as mistranslation, cause variation in the amino acid sequences of proteins that are

translated from the same mRNA transcript. **d |** Epigenetic modifications, such as the yeast prion [*PSI*+], cause variation in protein sequences, in this example via stop-codon readthrough.

Figure 2 | **Robustness causes evolvability by providing access to a diversity of mutational neighbourhoods. a,b** | The mutational neighbourhoods of the ancestral steroid receptor (AncSR1 in ref [91]; part **a**) and the derived steroid receptor after 11 amino acid changes (AncSR1+11p in ref [91]; part **b**). Each vertex (circle) corresponds to a sequence of amino acids at four sites in each protein's recognition helix: the three that historically changed binding specificity, plus an adjacent site. Of all 160,000 possible such sequences in each background, only functional sequences are shown — i.e., sequences that bind the oestrogen (pink) or the steroid (blue) response elements, or that promiscuously bind both (yellow). Edges connect sequences that differ in a single amino acid. The number of functional sequences differs dramatically between the two backgrounds: 129 in the ancestral background, as compared to 1,351 in the derived background. **c,d** | Moreover, the lengths of the shortest paths from a sequence that binds the oestrogen response element to a sequence that binds the steroid response element is much longer in the ancestral background (part **c**) than in the derived background (part **d**). The * symbol indicates starting points from which there is no path to a sequence that binds the steroid response element. Data from ref [91]. **[Copy Ed: no credit line is needed for actual figure adaptation. Although the data are derived from Ref91, the figures themselves are not from there (or even from the supp info of the original article). It's also Nature anyway, so no formal copyright clearance would be needed anyway.]**

Figure 3 | **Adaptive landscape topography influences evolvability. a |** A smooth, single-peaked landscape facilitates evolvability, because mutations can create adaptive phenotypic variation from anywhere in the landscape, except atop the global peak. For example, the white and black circles denote two distinct mutational paths that start from different points in the landscape, but that both converge on the global peak via a series of 'uphill' mutational steps. **b |** By contrast, a multi-peaked, or rugged landscape hinders evolvability, because an evolving population may become trapped on local, suboptimal peaks. For example,

whereas the mutational path indicated by the white circles leads to the global peak, the mutational path indicated by the black circles does not. **c |** The shape of an adaptive peak is a consequence of magnitude epistasis. Specifically, positive epistasis generates peaks that are convex, whereas negative epistasis generates peaks that are concave. As a population climbs an adaptive peak, evolvability tends to increase if the peak is convex, whereas it tends to decrease if the peak is concave. **d |** Landscape ruggedness is a consequence of sign epistasis, which creates adaptive valleys that may be difficult for an evolving population to cross. Grey circles correspond to those in part **b**. **e-g |** The same landscape as in part **a**, but shown as two-dimensional contour plots. Open circles indicate genotypes and edges connect genotypes that differ by a single mutation. The same landscape can be studied by: systematically engineering genotypes that contain all possible combinations of a small number of mutations (part **e**); deep mutational scanning of a single wild-type genotype, including all single-mutants, many double-mutants, and some triple-mutants (part **f**); or in the case of small landscapes, via the exhaustive enumeration of all possible genotypes (part **g**).

Glossary

Isogenic populations

Populations of individuals with the same genotype.

Phenotypic plasticity

The ability of one genotype to produce more than one phenotype in response to different environmental stimuli.

Modularity

The extent to which a system can be partitioned into distinct components.

Pleiotropy

When one gene or one mutation affects multiple phenotypes.

Pre-mutation evolvability

Evolvability driven by new mutations.

Post-mutation evolvability

Evolvability driven by existing genetic variation within a population, for example via recombination acting on that variation.

Gene expression noise

Variability among isogenic cells in transcript or protein abundance.

Viral latency

The ability of a virus to remain dormant in a host cell.

Competence

The ability of a cell to take up DNA from the environment.

Tolerance

The ability of bacteria to survive in the presence of antibiotics without developing resistance.

Population bottleneck

A temporary, drastic reduction in population size.

Genetic assimilation

A process by which a new phenotype that results from an environmental perturbation becomes genetically encoded.

Kinetic trapping

Occurs when a protein does not reach its minimum free-energy structure, but rather becomes trapped in a non-equilibrium structure.

Stop-codon readthrough

When translation does not terminate at a stop codon, but rather continues to extend an amino acid chain.

Prions

Proteins that propagate by inducing properly folded proteins to convert into a misfolded form, often resulting in aggregation.

Cryptic genetic variation

Genetic variation that normally causes little to no phenotypic variation, but that has the potential to cause phenotypic variation in new environments or new genetic backgrounds.

Enhancer

A short DNA sequence that is bound by regulatory proteins to activate the transcription of a gene, which may be located many thousands of base pairs away.

Chaperones

Proteins that assist other proteins in folding, or refold misfolded proteins.

Epistatic interactions

Non-additive interactions between alleles in their contribution to a phenotype or fitness.

Protein domain

A distinct functional and often autonomously folding unit of a protein.

Genotype space

The space of all possible genotypes. For a nucleic acid sequence of length L, this space comprises 4^L genotypes.

Concave

A real-valued function on an interval of real numbers is concave if any line connecting two points on the graph of the function lies on or below the graph.

Convex

A real-valued function on an interval of real numbers is convex if any line connecting two points on the graph of the function lies above or on the graph.

Adaptive walks

A series of mutations that never decrease fitness.

Saddle points

Points on a landscape that have zero slope in at least two orthogonal directions, yet are not local peaks.

Extra-dimensional bypasses

Accessible mutational paths to an adaptive peak that are faciltated by increasing the dimensionality of an adaptive landscape.

Quantitative trait loci

Loci that explain part of the genetic basis of variation in a phenotype.

Key points

- Evolvability is the ability of a biological system to produce phenotypic variation that is both heritable and adaptive.
- Recent technological advances are transforming evolvability research from a field dominated by theory to one illuminated by experiment.
- We highlight three causes of evolvability that have been the focus of recent experimental research. They are phenotypic heterogeneity, robustness, and adaptive landscape topography.
- We discuss the mounting evidence that these causes of evolvability can evolve, and also the question of whether they can evolve adaptively.

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Author contributions

J.L.P. and A.W. contributed equally to all aspects of this work: researching and discussing content, writing the manuscript and reviewing/editing it before submission.

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Competing interests

The authors declare no competing interests.

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In this article, Payne and Wagner discuss how recent experimental studies are complementing theoretical work to enhance our understanding of the evolvability of diverse biological systems. They highlight phenotypic heterogeneity, robustness and adaptive landscape topography as causes of evolvability, and they additionally discuss evidence for whether evolvability itself can evolve.